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P01/7700 0.00-0218817/5

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The Patent Office

Cardiff Road  
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NP10 8QQ

1. Your reference

P014017.GB CLM

2. Patent application number

0218817.5

13 AUG 2002

Name, address and postcode of the or of each applicant (underline all surnames)

Fondazione Centro San Raffaele del Monte Tabor  
Via Olgettina, 60  
20123 Milano, Italy

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

7073455001

4. Title of the invention

Pharmaceutical Compositions

5. Name of your agent (if you have one)

D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

21 New Fetter Lane  
London  
EC4A 1DA

Patents ADP number (if you know it)

59006

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Country

Priority application number  
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Date of filing  
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Number of earlier application

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

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Description 35

Claim(s) 6

Abstract 1

Drawing(s) 5 + S

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77) 1 ✓

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature *D Young & Co* Date 13 August 2002  
D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom Catherine Mallalieu 020 7353 4343

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P014017GB

1

Pharmaceutical Compositions

Field of the Invention

- 5 The present invention relates to pharmaceutical compositions and to their use in anti-HIV vaccination.

Background of the Invention

- 10 The human retrovirus HIV (human immunodeficiency virus) has been demonstrated to be the causative agent of acquired immunodeficiency syndrome (AIDS), a disease for which there is currently no cure. Even though more than 20 years have elapsed since the first description of AIDS, no effective "preventive" vaccine is yet available against infection with HIV. The rapid rise in seropositivity among individuals in high risk  
15 categories, the virulence of the disease, and its growing world-wide distribution, underscore an overwhelming and immediate need for a vaccine capable of inducing complete protective immunity in non-infected individuals.

- Induction of a *bona fide* protective immunity could be achieved by a vaccine that  
20 elicits the production of viral antigen-specific antibodies targeted at the external coat of the virus (envelope) which are capable of neutralizing the early events of the viral life cycle (i.e., attachment to the cellular membrane, fusion and entry). However, all the experience accrued in this field over a period of more than a decade has clearly demonstrated that the development of such a vaccine represents an extraordinary  
25 challenge.

- HIV encodes an envelope glycoprotein precursor, gp160, which is proteolytically cleaved into the exterior (gp120) and transmembrane (gp41) glycoproteins (Allan et al., 1985; Kowalski et al., 1987). The gp120 glycoprotein remains associated with the  
30 mature envelope glycoprotein complex through a non-covalent interaction with the gp41 ectodomain (Veronese et al., 1985).

Current strategies for developing vaccines against infection by HIV have focused on eliciting antibodies against the external viral envelope glycoprotein gp120 or its cell surface receptor CD4. However, attempts to generate anti-HIV-1 vaccines based on  
5 purified, soluble forms of gp120 have been unsuccessful. Reasons for this failure include, firstly, the extraordinary propensity of gp120 to mutate its primary and secondary structure, thereby making the virus a continuously "moving target", and, secondly, the concealment of critical neutralization epitopes expressed on the envelope surface by locating them inside deep surface cavities or by presenting them in cryptic  
10 form (Kwong et al., 1998).

New hope was raised by the observation that some of the cryptic conserved neutralization epitopes on the gp120 glycoprotein, specifically those involved in coreceptor binding (Thali et al., 1993), as well as others, including the principal  
15 neutralization domain or "V3 loop" (Sattentau and Moore, 1991; Mbah et al., 2001; Lusso and Burastero, unpublished data), become exposed and antibody-accessible upon binding of gp120 with the CD4 receptor, leading to virus-cell membrane fusion. Several groups have explored the possibility of immunizing with a complex between gp120 and soluble CD4 (sCD4) in an attempt to present critical neo-epitopes to the  
20 immune system, which are unravelled by the receptor/envelope interaction (Celada et al., 1990; Kang et al., 1994; DeVico et al., 1995). More recently, one group has produced a single synthetic chimeric molecule encompassing both gp120 and a truncated CD4 molecule linked via a flexible linker that allows for intramolecular gp120-CD4 complex formation (Fouts et al., 2000).

25

The present invention identifies a problem associated with the aforementioned approaches. In its native form, the HIV envelope comprises an oligomeric complex of gp120 and gp41 subunits. The oligomeric complex is most likely a trimer comprising three gp120 and three gp41 subunits. This has important implications for the  
30 accessibility and conformation of several neutralisation epitopes (Yang et al., 2001): some of them are well exposed on the monomer but are not available to antibody-mediated neutralisation since they are hidden on the trimer (Sattentau et al., 1995;

Nyambi et al., 1998). Conversely, other epitopes may only be presented in the native oligomeric form. Thus, the use of soluble gp120-sCD4 complexes fails to present many of the most important neo-epitopes expressed by the HIV envelope protein during viral infection.

5

Importantly the associations between the six components of the fusion-competent envelope complex are relatively weak, making the complex unstable. Moreover, since the native complex falls apart before it can be purified, its use as an immunogen has been severely limited.

10

The concept of presenting to the immune system neo-epitopes of native HIV envelope that become visible at a transitional stage induced by interaction of the viral envelope with the receptor complex (CD4 and coreceptor) has been developed by LaCasse et al. (Science 1999), who used whole cells expressing the HIV envelope "frozen" (by chemical fixation) at random intermediate stages of fusion with cells expressing the receptor complex. However, a retraction of the published results revealed that only non-specific neutralisation due to an unknown cytotoxic effect of the complex sera was observed (Science 2002 May 10;296(5570):1025).

15

Thus, there remains a need to develop neutralising antigens that present to the immune system epitopes that are exposed during in-vivo attachment of HIV envelope protein to the receptor of target cells. The present invention overcomes this problem.

20

#### Summary of the invention

25

The invention relates to novel strategies for immunization against HIV. In particular, the invention provides for both 'in-vivo' and 'ex-vivo' generation of HIV envelope-CD4 complexes.

30

Thus, according to one aspect of the present invention there is provided a pharmaceutical composition comprising (i) a polynucleotide encoding HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor.

The polynucleotides are preferably encoded on vectors. Preferably, the vectors are selected from the group comprising Murine Leukemia Virus, Lentiretroviruses, Herpesviruses, Adenovirus, Adeno-associated virus and Semliki Forest Virus.

5

The polynucleotide of (ii) may be encoded on the same or on a different vector to the polynucleotide of (i).

Thus in one aspect of the invention there is provided a pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor wherein (i) and (ii) are encoded on separate vectors.

In another aspect of the invention there is provided a pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor wherein (i) and (ii) are encoded on the same vector.

Alternatively, in another aspect of the invention, there is provided a pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a CD4 receptor.

The compositions preferably comprises several polynucleotides encoding HIV envelope proteins, each of which may originate from different HIV subtypes. These polynucleotides may exist on the same or on separate vectors.

In one embodiment the HIV envelope protein is expressed as part of a fusion protein and/or the CD4 receptor is expressed as part of a fusion protein.

In another embodiment, the CD4 receptor employed in the invention is in the form of a fusion protein.

Preferably the pharmaceutical composition according to the invention comprises a pharmaceutically acceptable diluent or excipient.

5 In a further aspect of the present invention there is provided a pharmaceutical composition of the present invention for use in the treatment of HIV.

In another aspect of the present invention there is provided a pharmaceutical composition of the present invention in the form of a vaccine.

10 In a further aspect of the present invention there is provided the use of a pharmaceutical composition of the present invention for the preparation of a medicament for use in generating anti-HIV-1 monoclonal antibodies.

15 In another aspect of the present invention there is provided the use of a pharmaceutical composition of the present invention for the preparation of a medicament for use in generating an immune response against HIV infection.

20 In a further aspect of the present invention there is provided a delivery system for (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor.

In a further aspect of the present invention there is provided a delivery system for (i) a polynucleotide encoding an HIV envelope protein and (ii) a CD4 receptor.

25 In a further aspect of the present invention there is provided a method of immunising a recipient against HIV infection comprising the administration of a pharmaceutical composition of the invention. In one embodiment, the administration of the pharmaceutical composition is in proximity of a superficial lymph-node station.

30 In the ex-vivo approach of the invention, HIV envelope-CD4 complexes are provided in the form of a fixed cell comprising HIV envelope protein complexed with a CD4 receptor.



Thus, according to a further aspect of the present invention there is provided a fixed cell wherein said cell expresses an HIV envelope protein and further wherein said envelope protein is complexed with a CD4 receptor.

5

In one embodiment, the fixed cell is derived from a T-cell.

In another embodiment the fixed cell is fixed with glutaraldehyde.

10 In a further aspect of the present invention, there is provided a fixed cell for use in the treatment of HIV.

In another aspect of the present invention there is provided a vaccine comprising the fixed cell of the invention.

15

In a further aspect another aspect of the present invention there is provided the use of a fixed cell of the invention for the preparation of a medicament for use in generating anti-HIV monoclonal antibodies.

20 In another aspect of the present invention there is provided an antibody immunospecific for the fixed cell of the invention.

In another aspect of the present invention there is provided the use of a fixed cell of the invention for the preparation of a medicament for use in generating an immune  
25 response against HIV infection.

In a further aspect of the invention there is provided a method of generating a fixed cell according to the invention comprising

- 30
- (i) expressing an HIV envelope protein on the surface of living cells;
  - (ii) complexing the envelope protein of (i) with a CD4 receptor; and
  - (iii) fixing the CD4 coated cells.

In a preferred embodiment the HIV envelope protein is expressed on the surface of the living cell by transducing the cell with a vector encoding the HIV-1 envelope protein.

- 5 In another embodiment the CD4 receptor is expressed from the living cell.

In another embodiment the living cell expresses the HIV envelope protein and the CD4 receptor from a single vector.

- 10 In another aspect of the present invention there is provided a pharmaceutical composition comprising at least one fixed cell of the invention and a pharmaceutically acceptable diluent or excipient.

- 15 In another aspect of the present invention there is provided a pharmaceutical composition comprising a population of fixed cells of the present invention, wherein within the population more than one HIV envelope serotype is expressed, and a pharmaceutically acceptable diluent or excipient.

- 20 In one embodiment the CD4 receptor as defined above in relation to certain aspects and embodiments of the invention includes soluble CD4 receptor.

Preferably, the CD4 receptor is the human CD4 receptor.

- 25 By "CD4 receptor" we include CD4 surrogate molecules including but not limited to M33 mini-protein.

The HIV envelope protein used in the present invention may be HIV-1 or HIV-2 and is preferably HIV-1 envelope protein.

- 30 In one embodiment the HIV envelope protein is the envelope protein of a primary HIV-1 subtype that utilises CCR5 coenzyme. CCR5-using isolates are the typical viral subtypes transmitted in vivo both in children (Scarlati et al., 1997) and in adults.

Preferably the polynucleotide encoding the HIV envelope protein of the present invention encodes HIV gp160.

5    Detailed description

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples.

- 10    The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory*  
15    *Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and*  
20    *Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; and E. M. Shevach and W. Strober, 1992 and periodic supplements, *Current Protocols in Immunology*, John Wiley &  
25    Sons, New York, NY. Each of these general texts is herein incorporated by reference.

The present invention provides a novel approach of presenting to the immune system neo-epitopes that are only transiently exposed during the process of viral envelope-CD4 interaction that leads to virus-cell fusion during HIV infection. A number of  
30    advantages of the present invention are that:

- the oligomeric conformation of the viral envelope is maintained and is

expressed on whole cells

- the viral envelope-CD4 complex can be generated *in vivo* improving the efficacy and specificity of the response
- the problem associated with the intrinsic instability associated with HIV envelope protein may be overcome
- vaccines comprising vectors are practical and easy to administer

Other advantages are discussed and are made apparent in the following commentary:

As used herein the terms “comprising” and “having” relate to not only to anything consisting of a specified feature or characteristic, but also anything including that feature or characteristic as well as one or more additional features or characteristics.

#### HIV envelope protein

Reference herein to HIV envelope protein includes variants, derivatives, analogues and homologues thereof, and polynucleotides encoding the same.

The native HIV envelope comprises a oligomeric structure composed of gp120 subunits and gp41 subunits. The receptor-binding (CD4 and coreceptor) sites are located in the gp120 moieties, and the fusion peptides are located in the gp41 components (Chan et al., 1997; Kwong et al., 1998; Kwong et al. (2000) J. Virol. 74:1961). The associations between the components of the fusion-competent complex are maintained via non-covalent interactions between gp120 and gp41 and between the gp41 subunits.

HIV cell entry is a multistep process that begins when gp120 engages CD4 on the cell surface. The two most common coreceptors used by HIV-1 are the chemokine receptors CCR5 and CXCR4 (Berger et al., 1997, Berger et al., 1998). Coreceptor binding by gp120 triggers a change in the conformation of gp41 that exposes its

hydrophobic fusion domain and permits insertion of the N-terminal peptide of gp41 into the cytoplasmic membrane of target cells (Weissenhorn et al., 1997; Chan et al., 1998; Chan et al., 1997). Further structural changes in this fusogenic form of gp41 then draw the virus and the cell membranes into close proximity to complete the fusion process. CXCR4 is the receptor for the chemokine SDF-1 (Bleul et al., 1996), whereas CCR5 serves as a receptor for the chemokines MIP-1  $\alpha$  and  $\beta$  as well as RANTES (Murphy, 1996).

The HIV envelope protein used in the present invention preferably comprises oligomeric gp120-gp41 complex. The oligomeric gp120-gp41 complex may be a trimeric complex comprising three gp120 subunits and three gp41 subunits.

The HIV envelope protein used in the present invention may be from HIV-1 or HIV-2 and is preferably HIV-1 envelope protein.

The polynucleotide employed by the invention to encode the HIV envelope protein preferably comprises the gp160 gene. The protein encoded by gp160 is proteolytically cleaved into the gp120 and gp41 glycoproteins intracellularly.

In another embodiment, the HIV envelope protein used in the present invention is a gp140 trimer comprising uncleaved ectodomains of gp120 and gp41. gp140 trimers may be stabilised by, for example, fusion with a C-terminal trimeric motif (Yang et al., 2002) or the addition of an intersubunit disulphide bond (Earl et al., 2002).

Preferably, the HIV envelope proteins are derived from a primary CCR5-dependent HIV-1 isolate, i.e., the predominate subtype that is transmitted from person-to-person *in vivo*.

HIV envelope proteins originating from different HIV subtypes may be used in the present invention. It will be understood by a skilled person that an effective vaccine to HIV may provide protection against several serotypes. Thus, a pharmaceutical composition or vaccine according to the invention may include polynucleotides that

encode for more than one HIV envelope protein from different HIV serotypes. A pharmaceutical composition or vaccine of the invention may also include a fixed cell according to the invention that comprises CD4 complexed to HIV envelope proteins from different HIV serotypes. Alternatively, a pharmaceutical composition or vaccine  
5 of the invention may include more than one fixed cell, each fixed cell comprising CD4 complexed to HIV envelope protein from a different HIV serotype.

Of the two major HIV types, HIV-1 and HIV-2, HIV-1 is the most virulent and is the predominant species around the world. To date, two major groups of HIV-1 exist, "M"  
10 and "O". The virus that causes the great majority of HIV-1 infections are in the M group. The O group isolates are genetically quite distant from the M group. HIV-1 subtypes of the M group include subtypes A-J. Subtype A is the most genetically diverged subtype. Subtype A is mostly prevalent in Central Africa, but has also been found in other parts of the world, including Europe, Russia, East Asia and America.  
15 Subtype B represents the vast majority of subtypes circulating in the western world. Subtype E has also entered the American continent, including the USA, and subtype E variants have been found in Central Africa and Asia. Subtype C seems to have its major centre in East and South Africa, but has been reported to occur in many other parts of the world, including Europe, Russia, China, India, and Brazil.

20

Examples of HIV envelope protein sequences are readily available from commercial and institutional HIV sequence databases, such as GENBANK, or as published compilations.

## 25 CD4 Receptor

Reference herein to CD4 receptor include variants, derivatives, analogues and homologues thereof, and polynucleotides encoding the same.

30 The CD4 receptor belongs to the immunoglobulin superfamily (Maddon et al., 1985) with four extracellular domains (D1-D4). The gp120 binding site is contained in the N-terminal D1 domain. In the CD4-gp120 complex, the CD4 surface binding to gp120 is

centered on the CDR2-like loop, a protruding  $\beta$ -hairpin with its C'  $\beta$ -strand forming an antiparallel  $\beta$ -sheet with the gp120  $\beta$ 15 strand. The major binding epitope for gp120 appears to be between residues 40-60 (Peterson and Seed, 1988; Arthos et al., 1989), which is homologous to the CDR2 site of the immunoglobulin light chain.

5 Residues 43 and 59 are particularly important for gp120 recognition with the Phe43 side chain occupying the entrance of the gp120 cavity, and the Arg59 side chain forming a double H-bond with the gp120 Asp368 side-chain (Kwong et al., 1998; Kwong et al., (2000) *Structure Fold. Des.* 8:1329). Another domain at residues 80-100 resembling CDR3 of Ig has also been considered to be important either for gp120

10 binding or for events leading up to fusion after initial binding (Camerini and Seed, 1990; Ohki et al., 1990; Lifson et al., 1991; Truneh et al., 1991).

In one embodiment, the CD4 receptor used in the invention is soluble human CD4 receptor. Soluble CD4 receptors are well known in the art.

15

In another embodiment, the CD4 receptor used in the invention is human CD4 receptor.

A truncated form of soluble CD4 which is smaller than the wild-type CD4 and well

20 suited for expression in living cells, may be used in the present invention. An example of such a molecule is described by Berger (1988) *Proc Natl Acad Sci U S A* 85:2357. Alternatively, other molecular forms, modified mutants or CD4-mimicking molecules based or not based on CD4 sequences may be used.

25 A surrogate CD4 molecule may be used in the present invention. Surrogate CD4 molecules may exhibit all major functional characteristics of native human CD4, while offering important advantages such as small size and high stability. Surrogate molecules employed by the invention may include CD4 miniproteins such as those described by Vita et al., *Proc. Natl. Acad. Sci. U S A* 96:13091 and the M33 mini-

30 protein described by Vita et al., in press, may be used in the present invention.

CD4 or CD4-mimetic agents can be administered as pre-formed proteins, produced by chemical synthesis or recombinant technologies, or alternatively, they can be engineered into plasmid DNA or viral vectors, or other forms of gene delivery systems. Such delivery systems may be used to drive expression *in-vivo* at the site of delivery.

### Polynucleotides

Polynucleotides used in the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides used in the invention to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed. The polynucleotides may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

The polynucleotides used in the invention may encode fusion proteins for example to aid in cellular secretion of the expressed polypeptides. Where, for example, two polypeptides of the invention, e.g., HIV envelope protein and CD4 receptor, are desired to be expressed from the same cell, it may be desirable to add targeting sequences to target the proteins to particular cell compartments or to secretory pathways. Such targeting sequences have been extensively characterised in the art.

Polynucleotides such as DNA polynucleotides may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

30

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve



making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating  
5 the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

#### 10 Protein

As used herein, the term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. As used herein, the terms "polypeptide" and  
15 "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulfide bonds. The terms subunit and domain may also refer to polypeptides and peptides having biological function.

#### Variants, Derivatives, Analogues, Homologues and Fragments

20

In addition to the specific proteins and nucleotides mentioned herein, the present invention also encompasses the use of variants, derivatives, analogues, homologues and fragments thereof.

25 In the context of the present invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be modified by addition, deletion, substitution modification replacement and/or variation of at least  
30 one residue present in the naturally-occurring protein.

The term "derivative" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of and/or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide retains at least one of its endogenous functions.

The term "analogue" as used herein, in relation to polypeptides or polynucleotides includes any mimetic, that is, a chemical compound that possesses at least one of the endogenous functions of the polypeptides or polynucleotides which it mimics.

Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required activity or ability. Amino acid substitutions may include the use of non-naturally occurring analogues.

Proteins of use in the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the transport or modulation function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

"Fragments" are also variants and the term typically refers to a selected region of the polypeptide or polynucleotide that is of interest either functionally or, for example, in an assay. "Fragment" thus refers to an amino acid or nucleic acid sequence that is a portion of a full-length polypeptide or polynucleotide.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

Polynucleotide variants will preferably comprise codon optimised sequences. Codon optimisation is known in the art as a method of enhancing RNA stability and therefore gene expression. The redundancy of the genetic code means that several different codons may encode the same amino-acid. For example, Leucine, Arginine and Serine

are each encoded by six different codons. Different organisms show preferences in their use of the different codons. Viruses such as HIV, for instance, use a large number of rare codons. By changing a nucleotide sequence such that rare codons are replaced by the corresponding commonly used mammalian codons, increased expression of the sequences in mammalian target cells can be achieved. Codon usage tables are known in the art for mammalian cells, as well as for a variety of other organisms. Preferably, at least part of the sequence is codon optimised. Even more preferably, the sequence is codon optimised in its entirety.

## 10 Vectors

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a host and/or a target cell for the purpose of replicating the vectors comprising the nucleotide sequences used in the invention and/or expressing the proteins used in the invention. Examples of vectors used in recombinant DNA techniques include but are not limited to plasmids, chromosomes, artificial chromosomes or viruses.

Polynucleotides used in the invention are preferably incorporated into a vector. Preferably, a polynucleotide in a vector for use in the invention is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

30

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the

control sequences more responsive to transcriptional modulators.

Vectors may be transformed or transfected into a suitable host to provide for expression of CD4 for use in present the invention. This process may comprise  
5 culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the protein, and optionally recovering the expressed protein.

The vectors used in the present invention may be for example, plasmid or virus vectors  
10 provided with an origin of replication, optionally a promoter for the expression of a polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, and/or a traceable marker such as GFP. Vectors may be used, for example, to transfect or transform a host cell.

15 Control sequences operably linked to sequences encoding proteins for use in the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal  
20 promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral  
25 or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of  $\alpha$ -actin,  $\beta$ -actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). Viral promoters may also be used, for example the Moloney murine  
30 leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

5

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

10

The vector used in the present invention may be a retrovirus based vector which has been genetically engineered so that it can not replicate and produce progeny infectious virus particles once the virus has entered the target cell. There are many retroviruses that are widely used for delivery of genes both in tissue culture conditions and in living organisms. Examples include and are not limited to murine leukemia virus (MLV), human immunodeficiency virus (HIV-1), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV) and all other *retroviridae* including lentiviruses. A detailed list of retroviruses may be found in Coffin *et al.*, 1997, "retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

25

Preferably, the vectors used in the present invention are selected from the group comprising Murine Leukemia Virus, Lentiretroviruses, Herpesviruses, Adenovirus, Adeno-associated virus and Semliki Forest Virus.

30

The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology. The natural target of

adenovirus is the respiratory and gastrointestinal epithelia, generally giving rise to only mild symptoms. Serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the young.

5

Viral gene expression can be divided into early (E) and late (L) phases. The late phase is defined by the onset of viral DNA replication. Adenovirus structural proteins are generally synthesised during the late phase. Following adenovirus infection, host cellular mRNA and protein synthesis is inhibited in cells infected with most serotypes.

10 The adenovirus lytic cycle with adenovirus 2 and adenovirus 5 is very efficient and results in approximately 10, 000 virions per infected cell along with the synthesis of excess viral protein and DNA that is not incorporated into the virion. Early adenovirus transcription is a complicated sequence of interrelated biochemical events but it entails essentially the synthesis of viral RNAs prior to the onset of DNA replication.

15

The organisation of the adenovirus genome is similar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied. Early cytoplasmic messenger RNAs are complementary to four defined, noncontiguous regions on the viral DNA. These regions are designated E1-E4. The  
20 early transcripts have been classified into an array of intermediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate regions.

The early genes are expressed about 6-8 hours after infection and are driven from 7 promoters in gene blocks E1-4.

25

Adenoviruses may be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters. The E1-replication defective virus may be propagated in a cell line that provides the E1 polypeptides in trans, such as the human embryonic kidney cell line 293. A  
30 therapeutic gene or genes can be inserted by recombination in place of the E1 gene. Expression of the gene is driven from either the E1 promoter or a heterologous promoter.

Even more attenuated adenoviral vectors have been developed by deleting some or all of the E4 open reading frames (ORFs). However, certain second generation vectors appear not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus.

An alternative approach to making a more defective virus has been to "gut" the virus completely maintaining only the terminal repeats required for viral replication. The "guttled" or "gutless" viruses can be grown to high titres with a first generation helper virus in the 293 cell line but it has been difficult to separate the "guttled" vector from the helper virus.

The adenovirus provides advantages as a vector for gene transfer for the following reasons:

It is a double stranded DNA nonenveloped virus that is capable of *in vivo* and *in vitro* transduction of a broad range of cell types of human and non-human origin. These cells include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated cells such as neurons.

Adenoviral vectors are also capable of transducing non dividing cells. This is very important for diseases, such as cystic fibrosis, in which the affected cells in the lung epithelium, have a slow turnover rate. In fact, several trials are underway utilising adenovirus-mediated transfer of cystic fibrosis transporter (CFTR) into the lungs of afflicted adult cystic fibrosis patients.

The expression of viral or foreign genes from the adenovirus genome does not require a replicating cell. Adenoviral vectors enter cells by receptor mediated endocytosis. Once inside the cell, adenovirus vectors rarely integrate into the host chromosome. Instead, it functions episomally (independently from the host genome) as a linear



genome in the host nucleus. Hence the use of recombinant adenovirus alleviates the problems associated with random integration into the host genome.

5 Pox viral vectors may be used in accordance with aspects of the present invention, as large fragments of DNA are easily cloned into its genome and recombinant attenuated vaccinia variants have been described (Meyer, *et al.*, 1991, J. Gen. Virol. 72: 1031-1038, Smith and Moss 1983 Gene, 25:21-28).

10 Herpes viral vectors may be derived from, for example, HSV1 or HSV2 strains, or derivatives thereof, preferably HSV1. Derivatives include inter-type recombinants containing DNA from HSV1 and HSV2 strains. Derivatives preferably have at least 70% sequence homology to either the HSV1 or HSV2 genomes, more preferably at least 90%, even more preferably 95%.

15 The use of HSV (Herpes Simplex Virus) strains in therapeutic procedures will require the strains to be attenuated so that they cannot establish a lytic cycle. In particular, if HSV vectors are to be used for therapy in humans the polynucleotide should preferably be inserted into an essential gene. This is because if a vector virus encounters a wild-type virus transfer of a heterologous gene to the wild-type virus could occur by  
20 recombination. However as long as the polynucleotide is inserted into an essential gene this recombinational transfer would also delete the essential gene in the recipient virus and prevent 'escape' of the heterologous gene into the replication competent wild-type virus population.

25 Attenuated HSV strains, here given as examples only, that may be used in the present invention include strains that have mutations in either ICP34.5 or ICP27, for example strain 1716 (MacLean *et al.*, 1991, J Gen Virol 72: 632-639), strains R3616 and R4009 (Chou and Roizman, 1992, PNAS 89: 3266-3270) and R930 (Chou *et al.*, 1994, J. Virol 68: 8304-8311) all of which have mutations in ICP34.5, and d27-1 (Rice and  
30 Knipe, 1990, J. Virol 64: 1704-1715) which has a deletion in ICP27. Alternatively strains deleted for ICP4, ICP0, ICP22, ICP6, ICP47, *vh*s or gH, with an inactivating mutation in VMW65, or with any combination of the above may also be used.

The terminology used in describing the various HSV genes is as found in Coffin and Latchman, 1996. Herpes simplex virus-based vectors. In: Latchman DS (ed). Genetic manipulation of the nervous system. Academic Press: London, pp 99-114.

5

The lentivirus group includes both "primate" and "non-primate" lentiviruses. Examples of primate lentiviruses include human immunodeficiency virus (HIV-1), the causative agent of human auto-immunodeficiency syndrome (AIDS), and simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the

10 prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

15 A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells. In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue. Thus, lentiviral vectors may advantageously be used in the present invention since

20 lentiviruses are capable of infecting a wide range of non-dividing cells, by contrast to certain other retroviruses that require cell division for stable integration.

The vectors used in the present invention may be specifically designed to carry bicistronic polynucleotide sequences coding for proteins used in the present invention.

25

#### Delivery Systems

The invention further provides a delivery system for (i) a polynucleotide encoding HIV envelope protein, and (ii) a CD4 receptor or a polynucleotide encoding the same.

30 (i) and (ii) may be delivered simultaneously or separately.

The delivery system of the present invention may be a viral or non-viral delivery system. Non-viral delivery mechanisms include but are not limited to lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof.

5

The polynucleotides may be delivered to the target cell population by any suitable Gene Delivery Vehicle, GDV. This includes but is not restricted to, DNA, formulated in lipid or protein complexes or administered as naked DNA via injection or biolistic delivery, viruses such as retroviruses, adenoviruses, poxvirus, lentiviruses, herpes  
10 viruses, vaccinia viruses, adeno associated viruses, murine leukemia viruses, semliki forest viruses and baculoviral viruses. Alternatively, the polynucleotides are delivered by cells such as monocytes, macrophages, lymphocytes or hematopoietic stem cells. In particular a cell-dependent delivery system is used. In this system the polynucleotides encoding the CD4 receptor and the HIV envelope protein are introduced into one or  
15 more cells *ex vivo* and then introduced into the patient.

The agents of the present invention may be administered alone but will generally be administered as a pharmaceutical composition.

## 20 Fixed Cells

As used herein, the term "fixed cell" refers to a cell that has undergone the process of fixation. The term "fixation" refers to a process that stabilises cell components and halts cell processes with a minimum alteration from the living state. Methods of cell  
25 fixation are well known in the art.

A fixed cell of the present invention may be derived from an animal cell that is capable of expressing HIV envelope protein on its surface.

30 Preferably, the animal cell is a cell of the immune system, e.g., a T-cell. Preferably the cell is a cell of the human immune system.

Antibodies

Antibodies may be produced by standard techniques, such as by immunisation using the polypeptides, polynucleotides, fixed cells and pharmaceutical compositions of the present invention or by using a phage display library.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, fragments produced by a Fab expression library, as well as mimetics thereof. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and F(ab')<sub>2</sub> fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies. Neutralising antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with the agents e.g., HIV envelope and CD4 receptor polynucleotides/polypeptides, fixed cells and pharmaceutical compositions of the present invention. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to epitopes of the HIV-envelope-CD4 complex contains antibodies to other antigens, the polyclonal

antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or  
5 humans.

Monoclonal antibodies directed against the agents of the present invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing  
10 cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

15 Monoclonal antibodies to the agents of the present invention may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al*  
20 (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al*  
25 (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

### 30 Treatment

It is to be appreciated that all references herein to treatment include curative, palliative

and prophylactic treatment of HIV related diseases. The treatment of mammals is particularly preferred. Both human and veterinary treatments are within the scope of the present invention.

## 5 Pharmaceutical compositions

A pharmaceutical composition is a composition that comprises or consists of a therapeutically effective amount of a pharmaceutically active agent. It preferably includes a pharmaceutically acceptable carrier, diluent or excipients (including  
10 combinations thereof). Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The  
15 pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Examples of pharmaceutically acceptable carriers include, for example, water, salt  
20 solutions, alcohol, silicone, waxes, petroleum jelly, vegetable oils, polyethylene glycols, propylene glycol, liposomes, sugars, gelatin, lactose, amylose, magnesium stearate, talc, surfactants, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, and the like.

25

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or  
30 ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or

subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestible solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

#### Vaccines

The preparation of vaccines which contain an immunogenic polypeptide(s)/polynucleotide(s) as active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-

alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

5

Further examples of adjuvants and other agents include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*),  
10 *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit,  
15 Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved for human use.

20

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture ( $\text{Al}_2\text{O}_3$  basis). Conveniently, the vaccines are formulated to contain a final concentration of  
25 immunogen in the range of from 0.2 to 200  $\mu\text{g/ml}$ , preferably 5 to 50  $\mu\text{g/ml}$ , most preferably 15  $\mu\text{g/ml}$ .

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic agent resulting from administration of this  
30 agent in vaccines which are also comprised of the various adjuvants.



The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

#### In-vivo immunogen formation

Animal cells may be transduced *in vivo* with a polynucleotide encoding an HIV-1 envelope protein and a polynucleotide encoding an sCD4 receptor, resulting in expression of native oligomeric HIV-1 envelope protein on the surface of transduced cells, as well as the local release of sCD4 receptor. The sCD4 receptor binds to the expressed envelope protein generating an immunogenic complex *in-vivo*.

Further preferred features and embodiments of the present invention will now be described by way of non-limiting example and with reference to the accompanying drawings in which:

Figure 1 shows a graphical output of a cytofluorimetric analysis using uninfected NIH-3T3 cells (panel A) or vCB43-infected NIH-3T3 cells in the presence or absence of sCD4 (panel B) as a target for serum IgG.

Figure 2 shows a graphical output of an ELISA assay using recombinant HIV-1 gp120 from a prototypic R5 isolate (JR-FL) or from a prototypic X4 isolate (IIIB), either alone or in combination with 2D-sCD4 as a target for serum IgG.

- 5 Figure 3 shows a plot of cell fusion activity, using NIH-3T3 mouse cells infected with vCB43 as effector cells and HeLa human cells infected with a vaccinia expressing human CCR5 (vCCR5) plus CD4 (vCB3) as a target, versus serum dilution.

Figure 4 shows a plot of cell fusion activity, using target cells expressing Env from different HIV-1 subtypes, versus serum dilution

10

Figure 5 shows a plot comparing cell fusion activity of serum IgG and serum IgG which has been pre-adsorption with human sCD4.

15 **Example 1 – Generation of serum IgG which preferentially recognises gp120-sCD4 complex**

Serum IgG obtained from mice injected subcutaneously with cells expressing a complex of gp120 and sCD4 on their surface preferentially recognize gp120 when complexed with sCD4, as shown by cytofluorimetric analysis (Figure 1). Pooled sera  
20 from 4 mice immunized with NIH-3T3 cells infected with a vaccinia vector expressing the HIV-1 BaL envelope (vCB43), then treated with 2D-sCD4 and fixed with glutaraldehyde were used. As a target for cytofluorimetric analysis uninfected (panel A) or vCB43-infected (panel B) NIH-3T3 cells were used. Solid lines represent the background fluorescence profile (cells reacted with FITC-conjugated, goat-anti-mouse  
25 IgG polyclonal antibody). Binding to cells treated (dotted line) or not (dashed line) with 2D-sCD4 are shown. The cells were analysed on a FACScan analyser.

**Example 2 – Generation of serum IgG which preferentially recognises solid phase recombinant HIV-1 gp120 solid phase antigen**

30

Serum IgG obtained from mice injected subcutaneously with cells expressing a complex of gp120 and sCD4 on their surface preferentially react with gp120/sCD4

complexes, as shown by ELISA assay (Figure 2). Pooled sera from 4 mice immunized with IgG from sera obtained from mice injected subcutaneously with NIH-3T3 cells infected with a vaccinia vector expressing the HIV-1 BaL envelope (vCB43) and treated with 2D-sCD4 were used. The solid phase antigen was recombinant HIV-1 gp120 from a prototypic R5 isolate (JR-FL) or from a prototypic X4 isolate (IIIB), used either alone or in combination with 2D-sCD4. The ELISA assay was performed using standard protocols.

### Example 3 – Inhibition of HIV-1 envelope-mediated cell fusion

HIV-1 envelope-mediated cell fusion is inhibited by pooled sera obtained from mice injected with NIH-3T3 cells expressing the HIV-1 BaL envelope (vCB43) and then treated with 2D-sCD4 (Figure 3). Sera from mice injected with cells expressing the HIV-1 envelope but not complexed with sCD4 were used as a control. To assess the relevance of anti-human CD4 antibodies to the observed neutralisation, sera from mice injected with a vaccinia vector expressing human CD4 (vCB3) were tested in parallel as a control. The cell fusion assay was performed as described (Feng et al. *Science* 1996) using NIH-3T3 mouse cells infected with vCB43 as effector cells and HeLa human cells infected with a vaccinia expressing human CCR5 (vCCR5) plus CD4 (vCB3) as a target. The sera, previously heat-inactivated, were added to target cells prior to the fusion assay at the dilution indicated. The results are expressed as percent fusion activity relative to the positive control (no inhibitor added).

### Example 4 – Inhibition of cell fusion mediated by diverse HIV-1 envelopes

Pooled sera from mice injected with NIH-3T3 cells expressing the HIV-1 BaL envelope (vCB43) and treated with 2D-sCD4 inhibit cell fusion mediated by diverse HIV-1 envelopes (Figure 4). Vaccinia recombinants encoding Env from different HIV-1 subtypes and with different coreceptor usage were used: vCB52, strain CM235 (Subtype A/E (Circulating Recombinant Form CRF01-AE); CCR5-dependent);

vCB43, strain BaL (subtype B; CCR5-dependent); vCB41, strain LAV (subtype B; CXCR4-dependent).

**Example 5 –Immune sera with CD4 independent activity**

5

Pre-adsorption with human CD4 does not abrogate the inhibitory activity of immune sera on HIV-1 envelope-mediated fusion (Figure 5). Pooled sera from 4 mice injected with NIH-3T3 cells expressing the HIV-1 BaL envelope (vCB43) were pre-adsorbed on Sub-B1 cells expressing high levels of human CD4. A drastic reduction of CD4  
10 reactivity was observed by ELISA.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing  
15 from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or  
20 related fields are intended to be within the scope of the following claims.

25

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**CLAIMS**

1. A pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor.
- 5 2. A pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor wherein (i) are (ii) are encoded on separate vectors.
- 10 3. A pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor wherein (i) and (ii) are encoded on the same vector.
- 15 4. A pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a CD4 receptor.
5. A pharmaceutical composition according to any one of the preceding claims wherein the HIV envelope protein is expressed as part of a fusion protein and/or the CD4 receptor is expressed as part of a fusion protein.
- 20 6. A pharmaceutical composition according to any one of the preceding claims wherein the CD4 receptor is soluble CD4 receptor.
7. A pharmaceutical composition according to any one of the preceding claims wherein the CD4 receptor is human CD4 receptor.
- 25 8. A pharmaceutical composition according to any one of the preceding claims wherein the CD4 receptor is a CD4 surrogate molecule.
9. A pharmaceutical composition according to claim 8 wherein the CD4 surrogate molecule is M33 mini-protein.
- 30

10. A pharmaceutical composition according to any one of the preceding claims wherein the HIV envelope protein is HIV-1 envelope protein.
11. A pharmaceutical composition according to any one of the preceding claims  
5 wherein the HIV envelope protein is CCR5-dependent HIV envelope protein.
12. A pharmaceutical composition according to any one of the preceding claims wherein the polynucleotide encoding the HIV envelope protein encodes gp160.
- 10 13. A pharmaceutical composition according to any one of the preceding claims further comprising a pharmaceutically acceptable diluent or excipient.
14. A pharmaceutical composition according to any one of the preceding claims for use in the treatment of HIV.  
15
15. A pharmaceutical composition according to any one of claims 1-14 in the form of a vaccine.
16. Use of a pharmaceutical composition according to any one of claims 1-15 for  
20 the preparation of a medicament for use in generating anti-HIV monoclonal antibodies.
17. Use of a pharmaceutical composition according to any one of claims 1-15 for the preparation of a medicament for use in generating an immune response against HIV infection.  
25
18. A delivery system for (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor.
19. A delivery system for (i) a polynucleotide encoding an HIV envelope protein  
30 and (ii) an sCD4 receptor.



20. A delivery system according to claims 18 or 19 wherein (i) and (ii) are administered simultaneously.
21. A delivery system according to any one of claims 18 to 20 wherein the CD4  
5 receptor is soluble CD4 receptor.
22. A delivery system according to any one of claims 18 to 21 wherein the CD4 receptor is human CD4 receptor.
- 10 23. A delivery system according to any one of claims 18 to 22 wherein the CD4 receptor is a CD4 surrogate molecule.
24. A delivery system according to claim 23 wherein CD4 surrogate molecule is M33 mini-protein.  
15
25. A delivery system according to any one of claims 18 to 24 wherein the HIV envelope protein is HIV-1 envelope protein.
26. A delivery system according to any one of claims 18 to 25 wherein the HIV  
20 envelope protein is CCR5-dependent HIV envelope protein.
27. A delivery system according to any one of claims 18 to 26 wherein the wherein polynucleotide encoding the HIV envelope protein encodes gp160.
- 25 28. A delivery system according to any one of claims 18 to 27 wherein the polynucleotides or polypeptides are administered orally, intracavernosally, intravenously, intramuscularly, subcutaneously, topically or by inhalation.
- 30 29. A method of immunising a recipient against HIV infection comprising the administration of a pharmaceutical composition according to any one of claims 1 to 15.

30. A method according to claim 29 wherein the administration of the pharmaceutical composition is in proximity of a superficial lymph-node station.

31. A fixed cell wherein said cell expresses an HIV envelope protein and further  
5 wherein said envelope protein is complexed with a CD4 receptor.

32. A fixed cell according to claim 31 wherein the CD4 receptor is soluble CD4 receptor.

10 33. A fixed cell according to claim 31 or 32 wherein the CD4 receptor is human CD4 receptor.

34. A fixed cell according to any of claims 31 to 33 wherein the CD4 receptor is a CD4 surrogate molecule.

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35. A fixed cell according to claim 34 wherein the CD4 surrogate molecule is M33 mini-protein.

36. A fixed cell according to any one of claims 31 to 35 wherein the HIV envelope  
20 protein is HIV-1 envelope protein.

37. A fixed cell according to any one of claims 31 to 36 wherein the HIV envelope protein is primary CCR5-dependent HIV envelope protein.

25 38. A fixed cell according to any one of claims 31 to 37 wherein the HIV envelope protein is gp160.

39. A fixed cell according to any one of claims 31 to 38 wherein the fixed cell is derived from a T-cell.

30

40. A fixed cell according to any one of claims 31 to 39 wherein the fixed cell is fixed with glutaraldehyde.

41. A fixed cell according to any one of claims 31 to 40 for use in the treatment of HIV.
- 5 42. A vaccine comprising the fixed cell according to any one of claims 31 to 41.
43. Use of a fixed cell according to any one of claims 31 to 41 for the preparation of a medicament for use in generating anti-HIV monoclonal antibodies.
- 10 44. An antibody immunospecific for the fixed cell according to any one of claims 31 to 41.
45. Use of a fixed cell according to any one of claims 31 to 41 for the preparation of a medicament for use in generating an immune response against HIV infection.
- 15 46. A method of generating a fixed cell according to the invention comprising
- (i) expressing HIV envelope protein on the surface of living cells;
  - (ii) complexing the envelope protein of (i) with a CD4 receptor; and
  - 20 (iii) fixing the CD4 coated cells.
47. A method according to claim 46 wherein said HIV envelope protein is expressed on the surface of the living cell by transducing the cell with a vector encoding the HIV envelope protein.
- 25 48. A method according to claim 46 or 47 wherein CD4 receptor is expressed from the living cell.
49. A method according to claim 48 wherein the living cell expresses said HIV envelope protein and said CD4 receptor from a single vector.
- 30 50. A pharmaceutical composition comprising a fixed cell according to any one of

claims 31 to 41 and a pharmaceutically acceptable diluent or excipient.

51. A pharmaceutical composition comprising a population of fixed cells according to any one of claims 31 to 41, wherein within the population more than one HIV envelope serotype is expressed, and a pharmaceutically acceptable diluent or excipient.

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ABSTRACT

PHARMACEUTICAL COMPOSITION

A pharmaceutical composition comprising (i) a polynucleotide encoding an HIV envelope protein and (ii) a polynucleotide encoding a CD4 receptor.

Figure 1

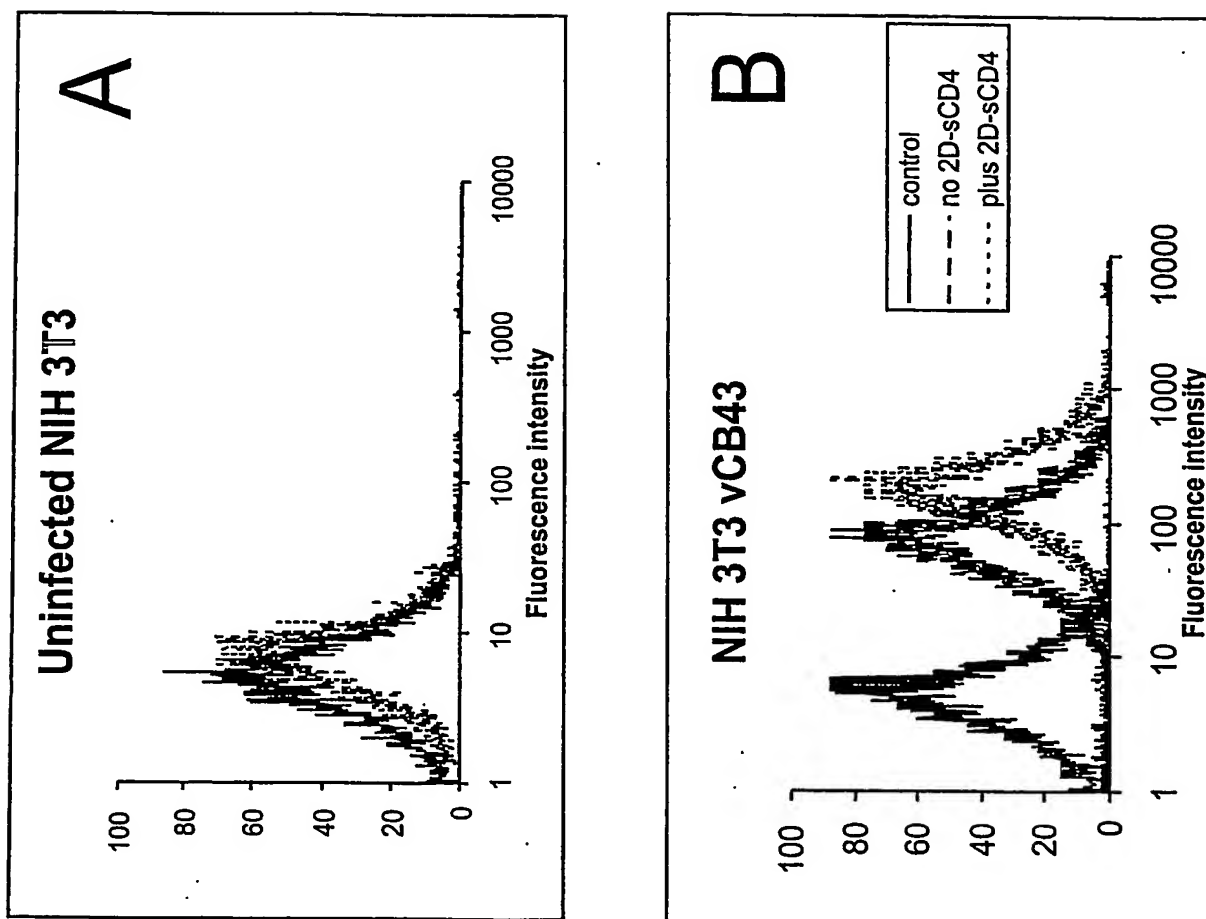
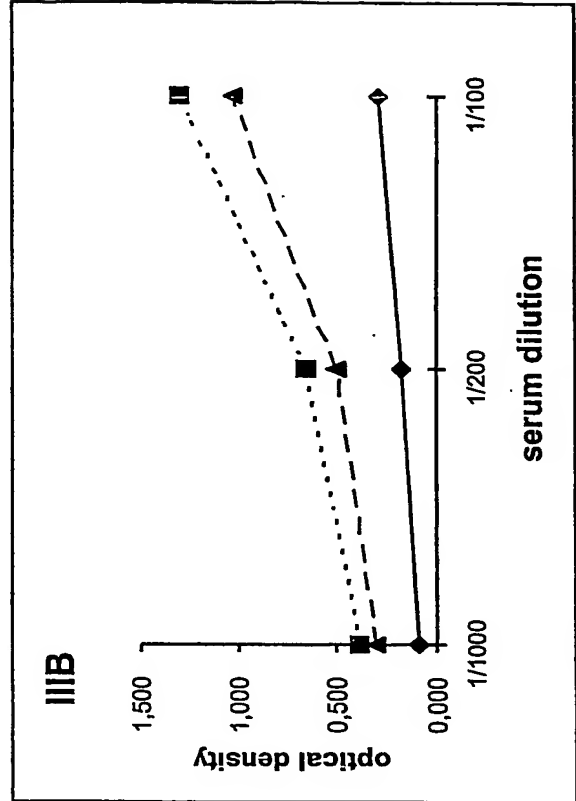
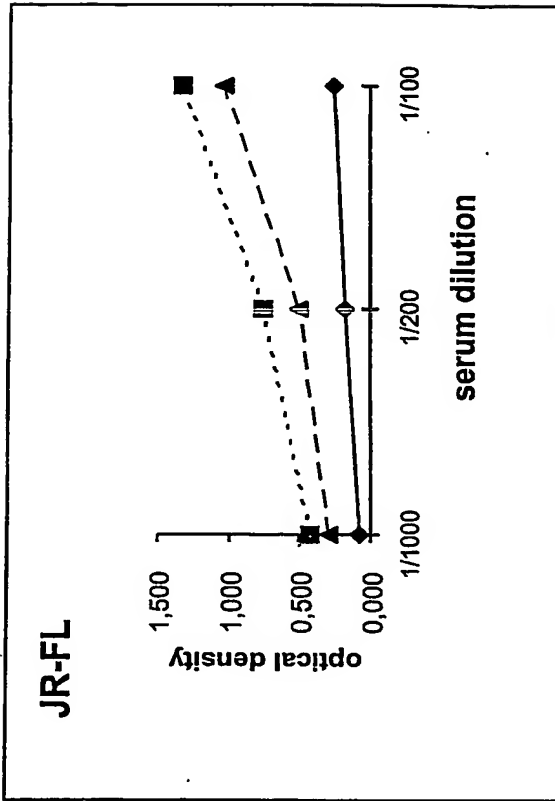
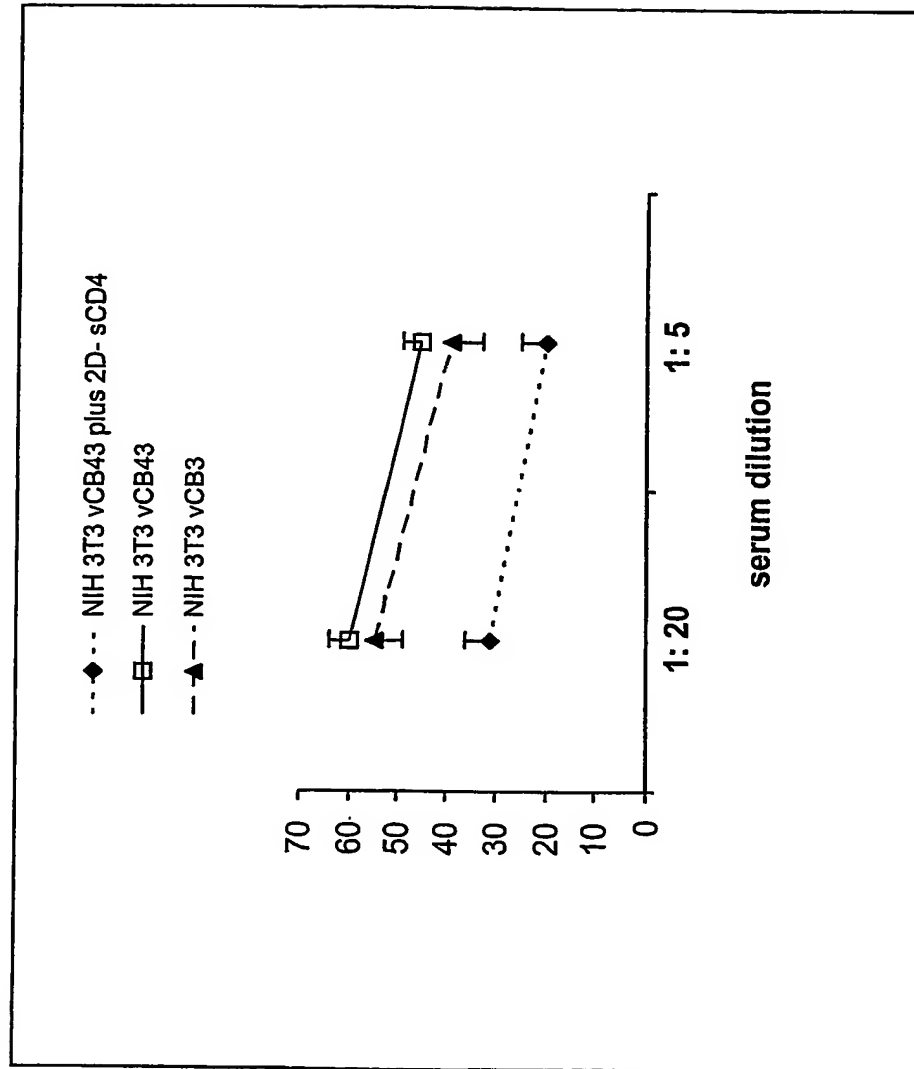


Figure 2

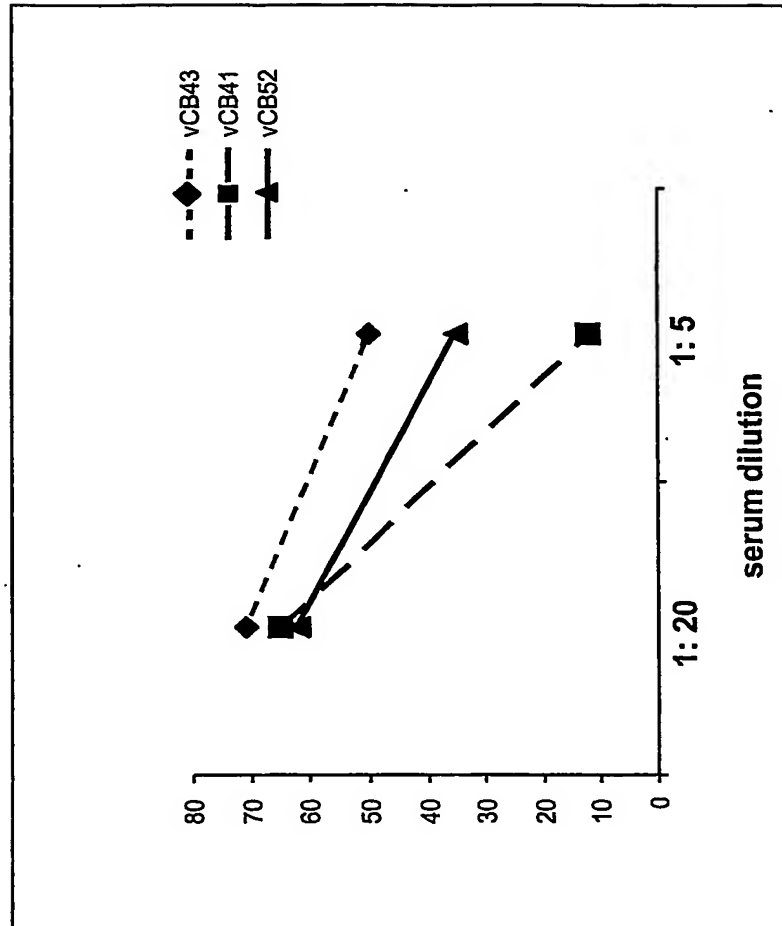


—◆— 2D-sCD4    - - -■- 2D-sCD4 + gp120    - -▲- gp120

Figure 3







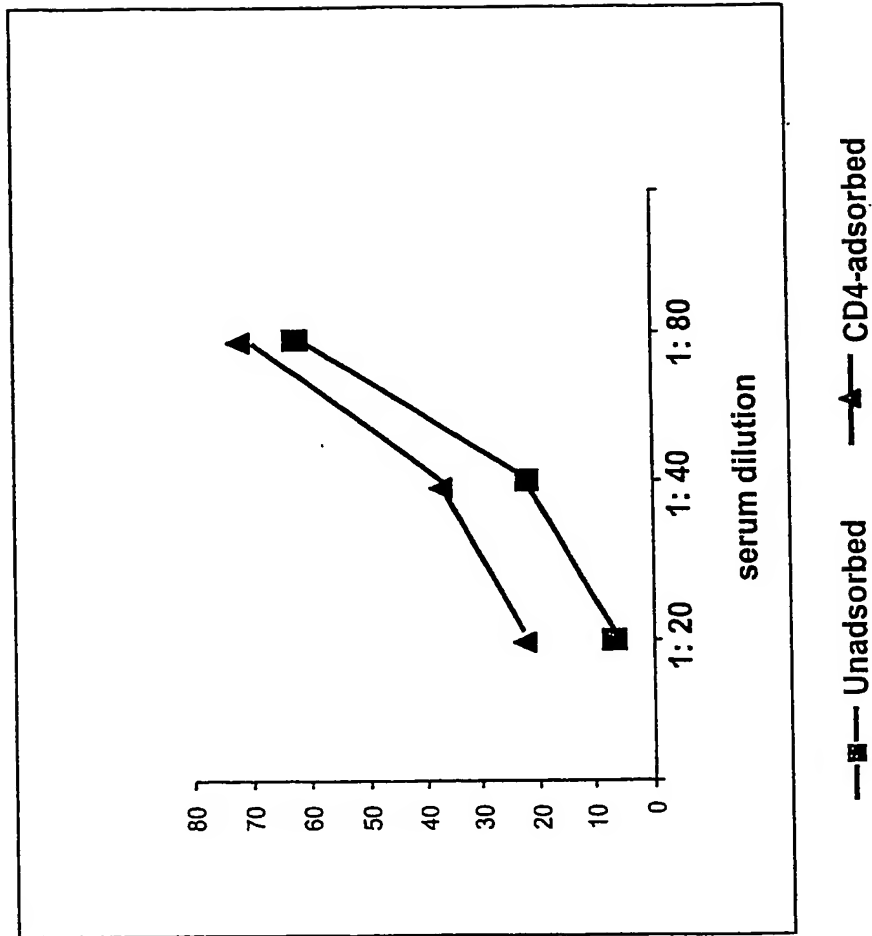


Figure 5

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